

Final Technical Report

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Center Name: NYU-EPA PM Center: Health Risks of PM Components

Center Director: Morton Lippmann

Title: Long Term Health Effects of Concentrated Ambient PM_{2.5}

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Research Category: Particulate Matter

Objective(s) of the Research Project: The Harvard Six Cities Study (Dockery, et al., 1993) and American Cancer Society (ACS) Cohort Study (Pope, et al., 1995) have shown substantially increased mortality in cities with higher average PM_{2.5} concentrations that was not explained by other risk factors. The validity and robustness of the Six Cities and ACS mortality studies findings were confirmed by the Health Effects Institute (HEI) Reanalysis project (Krewski, et al., 2000). The association between chronic exposure to PM_{2.5} and increased mortality was also significant when the ACS and Six Cities studies were extended for an additional 9 years (Pope, et al., 2001; Laden, et al., 2001). The PM effect size estimates reported in these studies are much larger than the cumulative effects reported for acute PM exposure and mortality (EPA, 2001). This finding indicates that people who live in areas with elevated PM experience cumulative adverse health effects in addition to acute transient effects. Increased mortality is not the only adverse health effect associated with PM exposure; several cross-sectional studies of children (Dockery, et al., 1989; Raizenne, et al., 1996) have shown that children who live in cities with higher average PM_{2.5} concentrations have more respiratory symptoms and decreased pulmonary function. Moreover, the Children's Health Study in Southern California has shown that chronic exposure to increased PM pollution is also associated with slower cumulative lung growth (Peters, et al., 1999a; b; Gauderman, et al., 2000; Avol, et al., 2001).

Based on these findings, the New York University (NYU) PM Center has conducted the first ever subchronic animal inhalation study using concentrated air particles (CAPs) in order to provide supplementary and complementary data analogous to that developed in the human cohort studies in cities with varying levels of fine PM. The studies began in 2002, with daily 6-hour exposures to CAPs for 5 days/week over a six month period. The main focus of this subchronic inhalation study was on the direct and indirect cardiopulmonary effects of PM. **This study tested the hypothesis that subchronic exposure of normal and compromised mice to CAPs will cause cumulative adverse affects on the respiratory and cardiovascular systems.**

The objectives of this study were to:

1. Determine the effects of subchronic CAPs exposure on pulmonary histopathology and lavage fluid biomarkers of lung inflammation and lung injury.
2. Determine whether subchronic CAPs exposure accelerates the development of atherosclerotic plaques in a mouse model of human atherosclerotic cardiovascular disease (apoE^{-/-} LDLr^{-/-} mice).

Summary of Findings:

Technical Aspects

Drs. Chen and Lippmann conducted the first subchronic animal inhalation screening study using FPM CAPs. A modified VACES (Maciejczyk, et al., 2005) was used to expose mice to at NYU's Sterling Forest Laboratory to a ten-fold concentration of Northeastern regional background FPM CAPs daily for 6 hr/d, 5 d/wk for up to 6 months. A cohort of C57BL/6 (C57) mice was used to investigate effects on the respiratory system. Other cohorts, i.e., C57 (n=6), and ApoE^{-/-} (ApoE knock-out) mice, implanted with electrocardiogram (ECG) transmitters (DataScience), were used to investigate the effects of FPM CAPs on the cardiovascular system. A separate cohort of double knockout mice (DK, ApoE and LDLr knockout) was also included to investigate histopathological changes and gene expression patterns of the cardiovascular and pulmonary systems. The overall mean concentration during the 30 hr/wk FPM CAPs exposure was $110 \pm 79 \mu\text{g}/\text{m}^3$ ($19.6 \mu\text{g}/\text{m}^3$ normalized annually). Detailed descriptions were published of the experimental design (Lippmann, et al., 2005a,b), modification of the exposure system (Maciejczyk, et al., 2005), and the observed effects in heart rate (HR), heart rate variability (HRV), atherosclerotic plaques on endothelia, gene expression, and brain cell distributions (Chen and Hwang, 2005; Chen and Nadziejko, 2005; Gunnison and Chen, 2005; Hwang, et al., 2005; Maciejczyk and Chen, 2005; Veronesi, et al., 2005), as well as an overall summary (Lippmann et al., 2005a). Brief descriptions of the study design and some key findings follow.

1. FPM CAPs Induced Alterations in HR and HRV. We used our recently developed non-parametric statistical method (Nadziejko, et al., 2004) to estimate the times that mean heart rates, body temperature, and physical activity differed significantly between the FPM CAPs and sham exposed groups. FPM CAPs exposure most affected HR between 1:30-4:30 AM, and the greatest effects were seen at the end of the 5-month exposure. We used a two-stage modeling approach to obtain the estimates of chronic and acute effects on these three response variables. As shown in Figure 1, there were significant decreasing patterns of HR (reaching a reduction of 33.8 beats/min in HR in FPM CAPs exposed ApoE^{-/-} mice compared to air exposed ApoE^{-/-} controls), body temperature, and physical activity in ApoE^{-/-} mice over the five months of FPM CAPs exposure, with smaller and non-significant changes in C57 mice (Hwang, et al., 2005). In addition, there was a 10 beats/min per $100 \mu\text{g}/\text{m}^3$ decrease in HR during the daily exposure period for the ApoE^{-/-} mice that was not seen in the C57 mice.

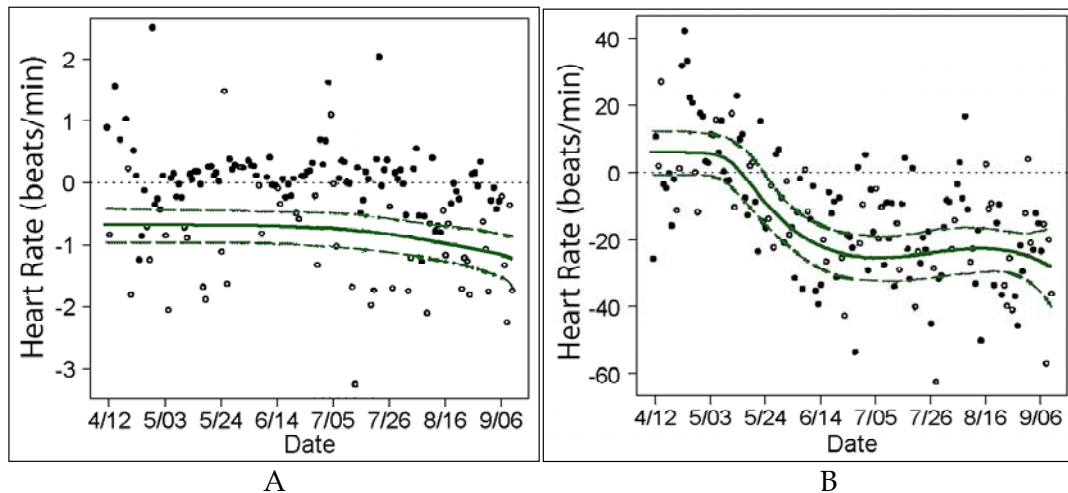


Figure 1. A: The Posterior Means (Solid) and 95% Equal-Tail Credible Intervals (Dotted) of HR During Exposure (Between 11:00-13:00); and B: Between 1:00 and 4:00 AM for ApoE^{-/-}. The circles in the plots are daily crude effects estimated in the 1st stage (full for exposure day and empty for non-exposure day).

At the same time, there was a quite different pattern of change for HRV (SDNN and RMSSD) (Chen and Hwang, 2005). There was a prolonged elevation, peaking at about two months into the study, a decline to below the initial levels by 4 months, and a relatively modest change in the last month of the exposure series. There were no HRV effects seen in normal C57 mice exposed to the same atmospheres. The response patterns indicated a perturbation of the homeostatic function in the cardiovascular system with initial stimulation (enhancement) and later depression of the HRV parameters. Bidirectional response to a low level pollutant challenge in a biological system is not uncommon. For example, the rate of clearance of inert particles from the lung conducting airways in response to sulfuric acid and cigarette smoke have been observed in both humans (Lippmann and Schlesinger, 1984; Lippmann, 2000) and in experimental animals (Chen and Schlesinger, 1983). A transient rise in HR was seen in the first month of FPM CAPs exposure, followed by a substantial decline in the second and third month, and a continued depression to the end of the exposure period. In addition, changes in HR fluctuation (HRF), a measure of variations in HR analogous to HRV, were markedly progressing at the end of the 5-month exposure period (Hwang, et al., 2005). Since the autorhythmicity of the cardiovascular system is modulated by many factors (Stauss, 2003), our results in HR, HRF, and HRV suggest that prolonged exposure to FPM CAPs may be necessary to alter the homeostatic function of the cardiovascular system. The need for prolonged FPM CAPs exposure to induce changes in these cardiac parameters also explained, at least in part, the absence of reported HRV alteration in many of the previous short-term studies involving animal exposures to FPM CAPs and other particles.

Using source apportionment analysis, four major FPM source categories were found in SF FPM CAPs, i.e., secondary sulfate (SS), resuspended soil (RS), residual oil (RO) combustion, and other, largely due to motor vehicle traffic (Maciejczyk and Chen, 2005). We then examined associations between these FPM components and both HR and HRV for three different daily time periods: during exposure, the afternoon after exposure, and late at night (Lippmann, et al., 2005b). For HR there were significant transient associations for RS during exposure, and for SS

in the afternoon after exposure. For HRV, there were comparable associations with RO in the afternoon after exposure and for both SS and RS late at night. The biologic bases for these associations and their temporal lags are not known but may be related to the differential solubility of the biologically active PM components at the respiratory epithelia and their access to cells that release mediators that reach the cardiovascular system. Clearly, further research to elucidate the underlying processes is needed. We will address this issue in our proposed HEI study by having more diverse FPM mixtures for exposure and, as we expect, greater variations in the responses. Using our daily measurements of the FPM components, source apportionments, and regression analyses, we expect to identify the FPM component(s) responsible for the acute and chronic changes in the biological measures of response.

In the 2nd subchronic study conducted between Feb. and May 2004, we investigated the effects of FPM CAPs exposure on the autonomic nervous system (ANS). In this study, we developed a new method to quantify the linear HRV parameters in a nonlinear Poincare plot (Li, et al., Submitted). We used two strains of mice that have been shown to be sensitive to PM exposure in terms of cardiac function changes, i.e., old AKR mice to model an elderly population susceptible to heart failure, and younger ApoE^{-/-} mice to model a younger population susceptible to atherosclerosis. Two exposure groups of each strain (n=8/group) were exposed to SF FPM CAPs or filtered air for 6 hr/d, 5 d/wk from Feb. 10th to May 7th, 2004. Ten second ECG, body temperature, and activity data were sampled throughout the study from each mouse every 5 minutes using implanted ECG transmitters.

By analyzing the baseline pre-exposure ECG waveform data for each mouse for 14 days, we were able to rank their mortality risk levels within each strain. We found that: 1) while no spontaneous deaths occurred in the ApoE^{-/-} group, those AKR mice that died spontaneously, or became very sick during the experiment (based on ECG criteria and visual observation) had higher baseline risk levels (based on their pre-exposure data); 2) exposure to FPM CAPs significantly increased AKR mortality risk; and 3) mice with higher baseline risk deteriorated faster than those with lower baseline risk during exposure to FPM CAPs.

2. *FPM CAPs Exposure Enhance Atherosclerotic Lesions.* The lungs, the hearts, the aortas, the brains, and the upper airways of all mice in the first FPM CAPs study were harvested for histopathological examination. For all mice, one lung was lavaged for biochemical and cellular endpoints. The other was perfused and stored in with 4% paraformaldehyde, embedded in paraffin, and serial sections prepared for hematoxylin and eosin staining. The heart and thoracic and abdominal aorta of the DK mice were removed *en bloc*, fixed in 4% paraformaldehyde and shipped to Dr. Douglas Taatjes of University of Vermont for quantitative immuno-histochemistry and morphometric analysis of the atherosclerotic lesions of the aorta roots (Chen and Nadziejko, 2005).

The cross sectional area of the aorta root of DK mice was examined morphologically using confocal microscopy for the severity of lesion, extent of cellularity, and lipid contents. Aortas from the arch to the iliac bifurcations were also sectioned longitudinally and lesion areas were stained with Sudan IV (Chen and Nadziejko, 2005). All DK mice, regardless of exposure, had developed extensive lesions in the aortic sinus regions, with lesion areas that covered more than 79% of the total area. In male DK mice, the lesion areas in the aortic sinus regions appeared to

be enhanced by FPM CAPs, with changes approaching statistical significance ($p=0.06$). In addition, plaque cellularity was increased by 28% ($p=0.014$) whereas there was no FPM CAPs associated changes in the lipid content in these mice.

When examining the entire aorta opened longitudinally, both the ApoE^{-/-} and DK mice had prominent areas of severe atherosclerosis covering 40% or more of the luminal surface. Visual examination of all images suggested that plaques tend to form in clusters concentrating near the aortic arch and the iliac bifurcations. Quantitative measurements showed that FPM CAPs exposure increased the percentage of aortic intimal surface covered by grossly discernible atherosclerotic lesion by 57% in the ApoE^{-/-} mice ($p=0.03$). Changes produced by FPM CAPs in male (10% increase) or female DK mice (8% decrease) were not statistically significant. Thus, subchronic exposure to FPM CAPs in mice prone to develop atherosclerotic lesions had a significant impact on the size, severity, and composition of aortic plaque. Effects of FPM CAPs on non-susceptible C57 mice were minimal.

In the 3rd subchronic study, in collaboration with Drs. Rajagopalan and Sun at Mt. Sinai School of Medicine, we confirmed that FPM CAPs exposure can indeed enhance atherosclerosis in ApoE^{-/-} mice and that the effects were dramatically enhanced by feeding mice with a high fat chow (HFC) (Sun, et al., 2005). As shown in Figure 2, at an average exposure concentration of 85 $\mu\text{g}/\text{m}^3$ (14.8 $\mu\text{g}/\text{m}^3$ normalized annually), in the FPM CAPs-HFC group, the mean composite plaque area was 41.5% vs. 26.2% in the filtered air (FA)-HFC group; while plaque area was 19.2% and 13.2% in the FPM CAPs-normal chow (NC) and FA-NC groups, respectively. Lipid content in the aortic arch as measured by oil red-O staining, revealed a 1.5 fold increase in FPM CAPs-HFC mice vs. the FA-HFC mice.

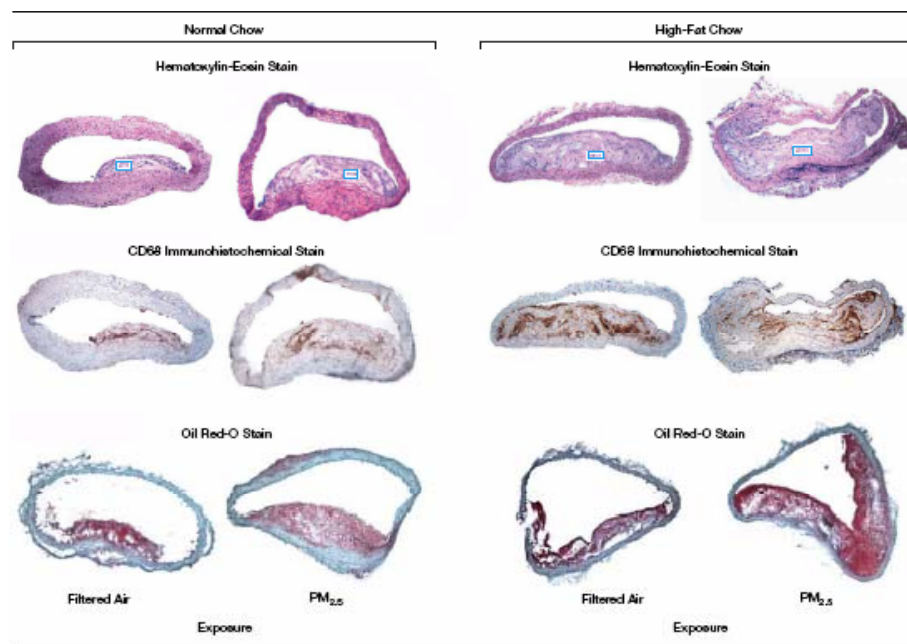


Figure 2. Representative Photomicrographs of Hematoxylin-Eosin Staining, CD68 Immunohistochemical Staining, and Oil Red-o Staining of Aortic Sections.

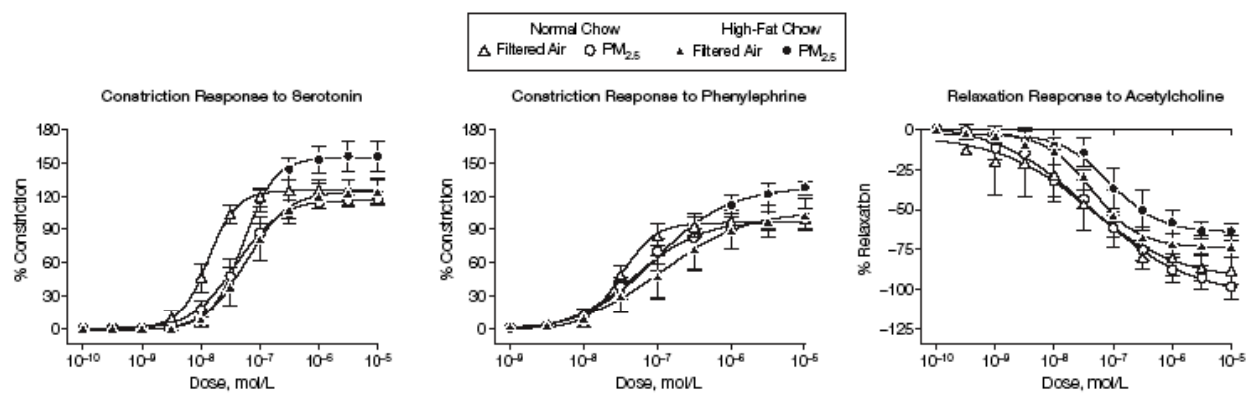


Figure 3. Mean Vasoconstriction of Aortic rings in Response to Serotonin and Phenylephrine, and Vasorelaxation in Response to Acetylcholine. Error bars represent SE. Values represent responses to graded doses of serotonin or phenylephrine expressed as a percentage of the peak response to 120 mEq/L of potassium chloride solution, or responses to graded doses of acetylcholine expressed as a percentage of precontracted tension in response to serotonin. For serotonin and phenylephrine, $P=0.03$ for mice exposed to CAPs and fed high-fat chow vs. other 3 groups. For acetylcholine, $P=0.04$ for half-maximal dose for dilation vs. all other groups.

In addition, FPM CAPs exposure also attenuates responsiveness to an endothelium-dependent agonist and heightens vasoconstrictor responsiveness. Figure 3 depicts responsiveness to the vasoconstrictors serotonin, phenylephrine, and the endothelium dependent agonist acetylcholine in thoracic aortic segments. Furthermore, vascular inflammation and protein nitration are prominent aspects of FPM CAPs-mediated effects on the vasculature. A 2.3-fold higher iNOS (inducible nitric oxide synthase) content was apparent in the FPM CAPs-HFC group compared with the FA-HFC group, and a 4.0 fold increase in the FPM CAPs-NC compared with the FA-NC group, whereas no significant difference was observed between the groups for eNOS (endothelial NOS) staining. In parallel with elevated iNOS expression, more 3-nitrotyrosine was detected in the plaque from FPM treated mice in both HFC mice and in NC mice.

Our results suggest that even seemingly low concentrations of PM_{2.5} exposure may have detrimental effects on the vasculature and bolster emerging data suggesting progression of carotid intima media thickening, a commonly used surrogate for atherosclerosis (Kunzli, et al., 2005). The concentration used in our study (although enriched) when normalized over a 24-hour/7-day period is well within the range of PM_{2.5} concentrations that individuals living in urban areas such as New York City are exposed to, and thus has implications for the long-term impact of FPM exposure on urban populations. Potentiation of atherosclerosis with FPM was noted in both the thoracic and abdominal aorta and was especially higher in response to high-fat feeding. Furthermore, the percentage increase in plaque burden with PM_{2.5} precisely paralleled the increase in macrophage and fatty infiltration noted in aorta, suggesting that these processes might be related. Thus, our most recent findings provide a potential biological basis for the association between atherosclerosis-related events noted in time-series analysis and prospective population cohort studies (Pope, et al., 2004, Peters, et al., 2004, Dockery, et al., 1993). In the proposed HEI study, we will expand our study to different areas of the U.S. having diverse FPM

compositions and, by using state-of-the-art source apportionment techniques, to identify the source categories or FPM components that are responsible for these effects.

3. *CAPs Induced Lesions in CNS.* In the 1st subchronic study, the brains of DK mice were preserved in 4% for subsequent histopathology of the brain. Microscopic examination of coronal sections of the brain, immunocytochemically stained for dopaminergic neurons, indicated that the number of neurons in the substantia nigral nucleus compacta were significantly reduced by 29% in FPM CAPs exposed ApoE^{-/-} relative to air exposed ApoE^{-/-} controls. In addition, statistical increases in astrocytes were noted. The dopaminergic neurons of the nucleus compacta is specifically targeted in Parkinson's disease. Our study expands the list of biological tissues affected by PM to include the brain and suggests an environmental role in the development of neurodegeneration in oxidative stress-susceptible individuals (Veronesi, et al., 2005).

4. *Gene Expression Levels of Lung and Heart Tissues.* At the termination of the 1st subchronic study, the lavaged lungs with the heart attached were removed and the tip of the heart was severed, frozen in liquid nitrogen, stored at -70°C and total RNA was extracted from these tissues, amplified, biotin-labeled and fragmented for hybridization and staining on Affymetrix mouse GeneChips® (430A). Data were normalized using the Robust Multiarray Average (RMA (Irizarry, et al., 2003)) method available in GeneTraffic™ (Iobion Informatics LLC) software and analyzed by the SAM (Significance Analysis of Microarrays) statistical technique (Tusher, et al., 2001) to identify genes that were up- or down-regulated in FPM CAPs-exposed mice relative to sham-exposed (control) mice (Gunnison and Chen, 2005).

Among the lists of heart and lung genes that might be affected by subchronic FPM CAPs exposure, the largest functional category is heat shock and other stress response genes. These genes respond to various stimuli such as elevated temperature, hypoxia, ischemia, hypothermia, free radicals, and certain chemicals. Several heat shock protein genes were down-regulated in FPM CAPs-exposed lungs (Dnaja1, Hspa8, Hsp105, Hspa1a, Hspa1b) and one of these (Hspa1b) also in heart tissue of exposed mice.

In addition to heat shock protein activity, certain other biological processes/molecular functions were affected by FPM CAPs exposures. Among these processes are DNA binding and regulation of transcription (Dbp, Cebpd, Sox4, Anp32a), defense responses (Ngp, Ccr2, Igh-6, Il1b, Igk-V5), proteolysis (Mmp8, Mmp9, Adam8), inflammatory response (Ccr1, Reg3g), and signal transduction and signaling pathways (Il1r2, Ccr1, Ccr2, Iigp, Agtrl1).

One gene, Dbp, that is up-regulated in the lung is of special interest because it has been associated with circadian rhythm, and there is evidence that PM exposure affects cardiac circadian rhythm. The Dbp gene is believed to be either a “clock-controlled” gene or a gene that regulates the output of the “clock”, i.e., the suprachiasmatic nucleus of the hypothalamus, which controls the circadian rhythm of physiological processes (Lopez-Molina, et al., 1997; Cheng, et al., 2002). As described earlier, we have shown evidence of perturbation of heart rate circadian rhythm due to FPM CAPs exposures. Therefore, the up-regulation of Dbp in lung tissue of two of the three FPM CAPs-exposed mice in this study merits closer evaluation to investigate a possible connection.

Most of the results of our 2nd (3 winter months) and 3rd (6 summer and fall months) subchronic CAPs exposure studies have not yet been published. In the first paper on results from our 3rd study, using our atherosclerotic mouse model, we showed that CAPs (av. = $85 \mu\text{g}/\text{m}^3$) enhanced atherogenesis in mice fed with a high-fat diet, with accompanying increases in lipid content, enhanced vasoconstrictor responses to phenylephrine and serotonin challenge in the thoracic aorta, attenuated relaxation to the endothelium dependent agonist against acetylcholine, and marked increases in macrophage infiltration, inducible isoform of nitric oxide synthase, generation of reactive oxygen species, and immunostaining for the protein nitration product 3-nitrotyrosine (Sun, et al., 2005). In $\text{ApoE}^{-/-}$ mice on a normal fat diet, some of these effects did not reach a level of statistical significance. Other analyses of results from this third subchronic CAPs inhalation study, i.e., on daily and long-term changes in cardiac function over the six-month exposure period in the $\text{ApoE}^{-/-}$ mice on a high fat diet, are included in a paper submitted to *Environmental Health Perspectives*.

During our analysis of the daily variations in cardiac function in our 3rd subchronic CAPs inhalation study, we noted the presence of a number of dramatic changes in cardiac function on certain days in the fall months. These observations led us to analyze the influence of daily variations in FPM component elemental concentrations on acute responses to ambient air FPM in terms of cardiac function in our mouse model of atherosclerosis. We found strong correlations with three metals (Ni, Fe, Cr) that generate reactive oxygen species (ROS).

Unusually high excursions of HR during November and December, 2004, not seen in the previous subchronic mouse CAPs inhalation studies, were noted, and we proceeded to examine the associations of HR and HRV with the FPM mass and elemental concentrations that were measured each exposure day. We found that the closest associations were with Ni and Cr, and that the days with high Ni and Cr had unusually low FPM mass concentrations. Figure 4 summarizes the differences between the 14 days with unusually elevated HRs and all of the other exposure days in terms of the exposure chamber concentrations of FPM, Al, S, V, Cr, Fe, Ni, Se, and Br, along with the average difference in HR and HRV between the CAPs exposed and air-sham exposed $\text{ApoE}^{-/-}$ mice. Assuming that the Ni, Cr, and Fe were associated with sulfate, they accounted for 12.4% of the FPM mass on those 14 days, and only 1.5% on the other days.

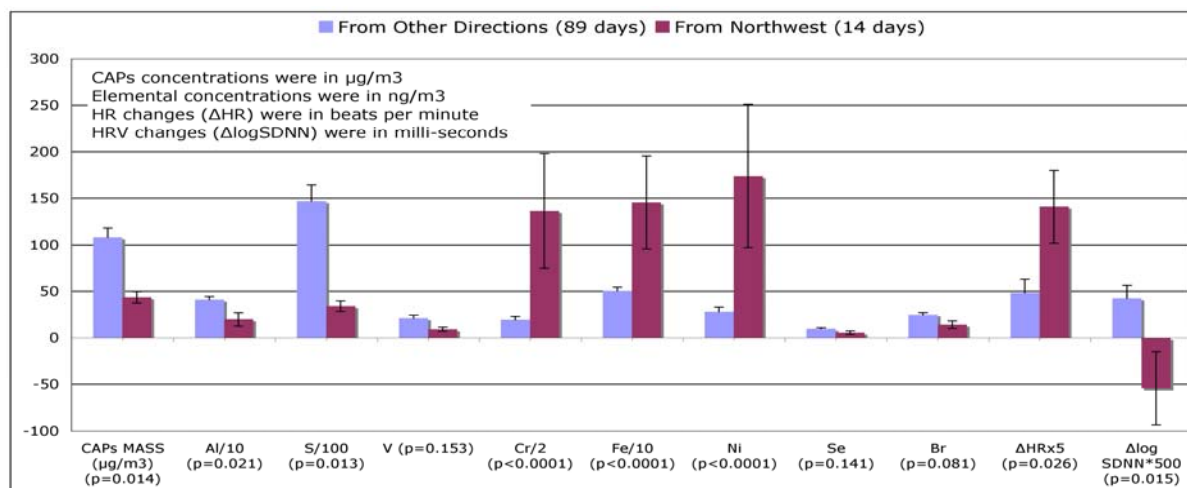


Figure 4. Average Elemental Concentrations and HR and HRV for 14 days When Winds were from the Northwest (right bar) and that for the 89 Days with Winds from all Other Directions and the Differences in Heart Rates of ApoE^{-/-} Mice Exposed to CAPs and Filtered Air. CAPs concentrations were in $\mu\text{g}/\text{m}^3$, elemental concentrations were in ng/m^3 , HR in beats/min, HRV (as log SDNN) in milliseconds. Error bars are \pm SE.

Back Trajectory Analyses. We next obtained back trajectory maps for the 14 days with the most notably elevated HRs, which all were associated with high-altitude winds from the northwest (See Figure 4). The 72-hour back trajectories from Sterling Forest for these 14 days appear to avoid population centers and industrial areas other than the Ni smelter near Sudbury, Ontario, which discharges its airborne effluents through a very tall stack.

The results reported here, from our third subchronic CAPs inhalation study in a mouse model of atherosclerosis indicate that inhalation exposure to Ni, more than V, is a more likely causal factor for the exacerbation of cardiac disease. If Ni inhalation, at current ambient air concentrations, does appreciably affect cardiac function and mortality in humans, the reader may wonder why it has not previously been recognized. One reason may be that the increment in cardiovascular mortality that Ni may produce is a relatively small part of the very large cardiovascular mortality. Also, the statistically significant transient and progressive changes that we have seen in cardiovascular function in our mice are relatively subtle, require advanced analytical techniques for their detection, and are unlikely to be detected in the kinds of short-term exposure studies that have previously been undertaken in laboratory animals.

In terms of environmental relevance, it is important to recognize that the peak Ni concentrations in the CAPs were only $\sim 175 \text{ ng}/\text{m}^3$ on the peak Ni exposure days, and only $26 \text{ ng}/\text{m}^3$ on the 89 other days, and there were no pronounced peaks for V (average $\sim 17 \text{ ng}/\text{m}^3$). Thus, Ni appears to be the component most likely to be causal for acute cardiac responses. The long-term average ambient air level of Ni in the U.S. is $1.9 \text{ ng}/\text{m}^3$, and the highest, in NYC, is $19 \text{ ng}/\text{m}^3$. Biological mechanisms that could account for the significant associations between Ni and the progression of cardiovascular disease in the mice, or with cardiovascular mortality in people exposed at low, environmentally relevant, ambient air concentrations is unknown, and warrants further, mechanistically oriented research in animals *in vivo* and cells *in vitro*.

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Relevant Web Sites: <http://www.med.nyu.edu/environmental/>
<http://es.epa.gov/ncer/science/pm/centers.html>